

FACTORS AFFECTING THE PRODUCTION OF LACTIC ACID BY MICROORGANISMS

BY

MURIEL HARDEN SVEC
B.A., University of Wisconsin, 1941
M.S., University of Wisconsin, 1943

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN
BACTERIOLOGY IN THE GRADUATE SCHOOL
OF THE UNIVERSITY OF ILLINOIS, 1947

CHICAGO, ILLINOIS

1947

FACTORS AFFECTING THE PRODUCTION OF LACTIC ACID BY MICROORGANISMS

BY

MURIEL HARDEN SVEC

B.A., University of Wisconsin, 1941

M.S., University of Wisconsin, 1943

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN
BACTERIOLOGY IN THE GRADUATE SCHOOL
OF THE UNIVERSITY OF ILLINOIS, 1947

CHICAGO, ILLINOIS

1948



The Effects of Some Metallic Ions on *Lactobacilli*

Muriel H. Svec¹

*From the Department of Bacteriology and Public Health, University of
Illinois, College of Medicine, Chicago, Illinois*

Received August 28, 1947

INTRODUCTION

Intensive studies on the nutritional requirements of the organisms used in microbiological assays have led to a steady improvement in assay media. Relatively little attention, however, has been given to the adequacy of the inorganic constituents provided.

In 1945 Snell (1) reported that there had been no detailed investigation of the mineral nutrition of the lactic acid bacteria. Möller (2) showed manganese to be essential to the Orla-Jensen strain of *Lactobacillus plantarum*. Woolley (3) demonstrated the increased growth rate of *Lactobacillus casei* upon the addition of manganese to a peptone-glucose medium.

While this paper was being written, Bentley *et al.* (4) published a microbiological method for the determination of manganese based on the requirements of *L. arabinosus* for that ion, and MacLeod and Snell (5) demonstrated the essential nature of Mn, Mg and K for several lactic acid bacteria.

This study is concerned with an analysis of the inorganic composition of an assay medium and the effects of added ions on the growth and acid production of three assay organisms.

MATERIALS AND METHODS

The cultures used in this work were *Lactobacillus casei* 7469, *Lactobacillus arabinosus* 17-5, and *Lactobacillus delbrueckii* LD-5. Stocks were obtained from the American

¹ Present address: Department of Experimental Medicine, Northwestern University School of Medicine, 303 E. Chicago Avenue, Chicago, Illinois. This work is part of a thesis presented to the Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Type Culture Collection and transferred weekly on a Difco glucose-yeast extract agar to which were added 5 g. of liver extract/l.

The Landy and Dicken (6) medium for vitamin assay was chosen for the experimental work. Inocula, grown in this medium for 24 hours, were standardized by dilution to a transmittance of 50 at the 590 $m\mu$ setting on the Coleman spectrophotometer and were routinely employed in a concentration of 1% of the volume of medium to be inoculated. All cultures were incubated at 37°C. Turbidity was measured at 590 $m\mu$ with a Coleman spectrophotometer. Titratable acidity was determined on 10 ml. amounts of culture and expressed in terms of ml. *N*/10 NaOH necessary for neutralization.

Fermentations were carried out on 200 ml. quantities of media in Erlenmeyer flasks. Lactic acid was determined by the method of Friedemann and Graesser (7); residual glucose by the method of Somogyi (8). Volatile acid, alcohol, and acetone determinations were made, but the data are not reported because there was no significant difference between the experimental and control cultures. Fermentation balances were calculated for each flask.

Since the problem of contamination in trace metal analyses is a serious one, the warnings and recommendations of Sandell (9) have been heeded in these investigations. Only reagent grade chemicals were used. Diluting fluids were redistilled acids and ammonium hydroxide, as well as dithizone-treated organic solvents. Distilled water was redistilled through an all-glass apparatus. All glassware employed in analyses was Pyrex. The cleaning regime consisted of overnight contact with aqua regia, followed by alcoholic potassium hydroxide. The vessels were rinsed 5 times with distilled water and autoclaved. After cooling, they were rinsed 3 times with fresh glass-distilled water. Washings were tested with dithizone. A new porcelain evaporating dish was used for each ashing procedure. Reagent blanks were run routinely.

Two-liter quantities of Landy and Dicken medium were ashed at 500°C. Two ml. of concentrated sulfuric acid were added during the concentration of these solutions to prevent volatilization of metallic elements during the ashing. The ash was taken up in a mixture of dilute HCl and HClO₄. The resulting solution was analyzed for copper, cobalt, manganese, molybdenum, zinc, and magnesium according to the methods outlined by Parks *et al.* (10). Iron was determined by the dipyriddy method as modified by Kitzes *et al.* (11). The method recommended by Sandell (9) was used in estimating cadmium.

EXPERIMENTAL FINDINGS

a. Analysis of Landy and Dicken Medium for Metallic Ions

An effort was made to determine the quantities of various metals present in Landy and Dicken medium as it would be made up in an assay laboratory. While it is obviously impossible to duplicate conditions of contamination, or small errors in calibration of equipment, it was thought that some approximation of the amounts of metals likely to be present could be achieved. Table I shows the range of content, in mg./l., of 8 cations in 4 separate batches of medium.

TABLE I
Analysis of Landy and Dicken Medium for Metallic Ions

	Fe	Mn	Zn	Mg	Cd	Mo	Co	Cu
Mg./l added in Salts B of formula:	1.0	0.9		9.87				
Mg./l determined by chemical analysis:	1.35	2.25	1.03	10.52	0.022	0 ^a	0 ^a	0 ^a
	2.05	1.415	3.58	10.44	0.037	0	0	0
	1.12	2.67	3.14	16.05	0.027	0	0	0
	1.80	1.78	2.48	11.60	0.034	0	0	0.0013

^a Methods employed in these determinations are not sensitive to less than 1 γ .

It is apparent from these data that considerable amounts of iron, manganese, and magnesium are picked up in excess of those added in Salt Solution B of the formula. Molybdenum and cobalt are notably absent, or at least not detectable by the chosen method of assay. Copper tests were negative in four media out of five.

b. Copper

Copper sulfate was added to a batch of medium giving no test for that metal. The effect of the sulfate ion was controlled with sodium sulfate. Table II shows the effect of copper on the 3 lactobacilli. Addition of 0.01–0.1 mg. of Cu/l., a range expected to give stimulation if the organisms have an unsatisfied copper requirement, provided no significant increase in titratable acidity over the control medium. Figures shown in Table II are averages of results obtained on triplicate samples of each copper level and each incubation period. The concomitant turbidimetric and fermentation studies indicate that both growth and acid production were affected by the higher levels of copper. The organisms incubated 72 hours are, in some cases, able to overcome the inhibitory effect of the copper ion.

c. Cobalt

Hydrated cobalt chloride was added aseptically in amounts from 0.01–100 mg. to a medium which gave no test for the ion. Chloride effects were controlled with the sodium salt. Addition of small amounts

TABLE II
The Effect of Copper On Three Lactobacilli

Hours incub.	<i>L. casei</i>						<i>L. delbrueckii</i>						<i>L. arabinosus</i>					
	Tur- bidity		N/10 NaOH		Lactic acid		Tur- bidity		N/10 NaOH		Lactic acid		Tur- bidity		N/10 NaOH		Lactic acid	
	24	72	24	72	24	72	24	72	24	72	24	72	24	72	24	72	24	72
Cu																		
mg./l.																		
0	13	6	9.4	9.9	501	543	17	12	9.6	10	513	576	6	6	9.9	10	583	584.7
1.0	23	6	4.5	9.8	343	543	17	13	9.5	10	513	576	7	6	9.9	10	583	584.7
5.0	52	9	2.9	9.4	119	531	32	22	7.3	8.1	396	445	9	6	9.9	10	583	584.7
15.0	55	20	2.9	6.8	119	360	42	42	4.0	5.3	229	264						
20.0	88	39	1.5	3.0	46	119	61	89	2.1	2.8	126	153	14	7	9.7	9.9	579	584
25.0	100	100	0.3	0.3			100	100	0.3	0.3								
50.0													19	9	9.6	9.9	568	583
65.0													28	15	7.0	9.6	420	570
80.0													42	22	5.0	8.5	315	528.9
Uninoculated							100		0.3		0							

of the cobalt ion gave rise to no stimulation; increasing concentrations produced a decline in growth and acid production. As in the experiments with copper, turbidity, titratable acidity, and lactic acid reflected the same trend. For this reason, only turbidity data are given in Table III.

d. Molybdenum

Neutralized molybdenum pentachloride, added to a medium giving no test for Mo, produced no effect on acid production or growth of the 3 lactobacilli until the concentration exceeded 170 mg./l.

e. Zinc

Table I indicates that the zinc content of the media examined varied from 1 to 3.5 mg. of Zn/l. Accordingly, media with high and low zinc contents were selected for comparison. The effect of zinc sulfate added to the medium already containing 1 mg./l. is seen in Table IV. Slight stimulation resulted from the addition of 1 mg. or less zinc. This

TABLE III
The Effect of Cobalt on Lactobacilli

	<i>L. casei</i>		<i>L. delbrueckii</i>		<i>L. arabinosus</i>	
Hours incubation	24	72	24	72	24	72
Co.	Turbidity					
mg./l.						
0	12	12	22	13	7	7
20	15	12	34	15	7	7
40	42	14	52	13	15	7
50	85	23	77	51	66	7
60	91	48				
80	100	100	79	72	87	7
100			100	100	90	7
Uninoculated	100					

effect was not seen when zinc was added to the medium already containing 3.5 mg. The addition of 20 mg. or more produced a precipitate which probably contributed to rendering the medium unfavorable for the organisms.

TABLE IV
The Effect of Zinc on Lactobacilli as Shown by Titratable Acidity

Zn	<i>L. casei</i>		<i>L. delbrueckii</i>		<i>L. arabinosus</i>	
	Hours of incubation					
	24	72	24	72	24	72
mg./l.						
0 ^a	8.4	9.8	4.5	9.3	9.8	9.8
1.0	9.0	9.8	5.0	9.8	9.8	9.8
5.0	8.2	9.7	5.0	9.8	9.8	9.8
15.0	5.2	9.7	3.5	9.8	9.0	9.8
25.0	2.8	9.7	1.9	9.2	8.4	9.2
50.0	0.4 ^b	0.4	0.4	4.1	1.8	8.1
75.0					0.4	0.4

^a The medium was shown by analysis to contain 1.03 mg. of zinc/l.

^b A titration of 0.4 is equivalent to that of the uninoculated medium.

f. Cadmium

Table V shows the effect of addition of hydrated cadmium chloride to a medium containing 34.63 γ of Cd/l. After 24 hours incubation,

TABLE V

The Effect of Cadmium on Lactobacilli as Shown by Titratable Acidities

Cd ^a	<i>L. casei</i>		<i>L. delbrueckii</i>		<i>L. arabinosus</i>	
	Hours of incubation					
	48	72	48	72	48	72
<i>mg./l.</i>						
0 ^a	10.2	10.4	9.9	10.4	10.4	10.4
0.2	7.4	10.4	9.9	10.4	9.2	10.4
0.4	3.0	6.8	9.7	10.4	6.7	10.2
0.6	1.3	2.5	9.2	10.4	4.2	10.0
0.8	0.6	1.3	7.7	9.8	0.3	8.4
1.0	0.3 ^b	0.3	5.0	9.6	0.3	0.3
5.0	0.3	0.3	1.1	4.0		
10.0			0.3	0.3		

^a The medium contained 34.63 γ of Cd/l.

^b 0.3 ml. represents the titration of uninoculated medium.

only the control tubes showed turbidity; therefore, measurements were not made until 48 hours. This ion was more markedly inhibitory than the others tested.

g. Omission of Ions

Salt Solution B of the Landy and Dicken medium contains, in addition to sodium chloride, the sulfates of manganese, iron, and magnesium. When this solution is added to the medium in the required amounts, 1 mg. Fe, 0.98 mg. Mn, and 9.87 mg. Mg are supplied/l. Addition of larger amounts of these ions to the medium produced no stimulation.

In these experiments, the iron, manganese, and magnesium were omitted one by one, in pairs, and altogether. The effects of these alterations of 24-hour titratable acidities can be seen in Table VI. Four separate batches of medium are represented. Each figure in the table was obtained from triplicate samples on each batch of medium. The

TABLE VI

Effect of Variation in Salts B Added to Landy and Dicken Medium

Alteration of B salts	<i>L. casei</i>	<i>L. delbrueckii</i>	<i>L. arabinosus</i>
	Ml. N/10 NaOH above or below amount necessary for neutralization of complete medium		
-Mg and Mn	+0.8	+0.9	-0.35
-Fe and Mn	+0.66	+0.95	-0.8
-Fe and Mg	+0.6	+0.75	-0.25
-Fe	+0.2	+0.7	-0.4
-Mg	-0.2	-0.5	-0.1
-Mn	+0.17	+0.7	-0.6
-B Salts	+0.1	+0.2	-0.7

These figures are derived from triplicate samples on 4 separate batches of medium.

basic medium, prior to the addition of any of the ions contained in the B salts, averaged 488 γ of iron, 493.3 γ of manganese, and 7.45 mg of magnesium/l.

From Table VI it is apparent that the omission of ions from the medium was, in general, detrimental to *L. arabinosus*. The contaminating Mn and Mg were apparently sufficient to satisfy the requirements of *L. casei* and *L. delbrueckii* LD-5 (5). The acid produced by these two organisms was more under these conditions than when Mn and Mg were supplied according to the formula.

DISCUSSION

The question of whether or not the inorganic content of the medium may have an effect on the accuracy of vitamin assays prompted this investigation. In addition to the metals entering the medium from reagents and vessels, there is the possibility that certain ions may be incorporated into the medium in significant quantities along with the material being assayed. It is well known, for example, that the inorganic content of vegetation reflects that of the soil on which it is grown. Thus, it was thought possible that occasional high or low results in assays of certain foodstuffs might be attributable to ion content of the sample. In the light of the above results, in connection with the particular ions studied, this seems unlikely except in certain isolated instances. A survey of literature was undertaken to determine the mineral

content of 50 plant and animal tissues. The amounts of such tissues likely to be incorporated in assay tubes in the estimation of riboflavin were calculated, and from these figures were obtained the concentrations of zinc, copper, and cobalt which would thus be added to the medium. Only in the case of tomatoes containing 50 p.p.m. copper, and liver containing 245 p.p.m. zinc, both cited by Calvery (12), would the ion content of the sample have a possible effect on the results of that assay. Concentrations of manganese and iron would have to exceed 50 and 100 mg./l., respectively, before acid production would be affected adversely. These figures apply only to the Landy and Dicken medium.

Omission of certain ions of the B salts, as shown in Table VI, has produced some interesting results. Only the omission of magnesium and of all the B salts caused *L. casei* and *L. delbrueckii* to produce less acid. The increased acidity produced by the omission of magnesium and manganese, both of which seem to be necessary to the *Lactobacilli*, suggests the possibility that the Landy and Dicken formula does not provide an optimum ion balance for these organisms.

ACKNOWLEDGMENTS

The author wishes to thank Dr. Milan V. Novak and Dr. Ernst R. Kirch for aid and advice during the investigation and for reading the manuscript.

SUMMARY

1. The inorganic content of Landy and Dicken vitamin assay medium, as it might be made up in an assay laboratory, was determined. In addition to the Fe, Mn, and Mg supplied by formula, Zn and Cd were found.

2. Ions not detectable in the ash of the medium (Cu, Co, Mo) produced no stimulation of reproduction or lactic acid formation when added to the medium. Increased concentrations of Cu and Co inhibited cellular activity.

3. Reduction in lactic acid production caused by large quantities of cobalt and copper appeared to be a function of decreased reproduction of the cultures.

4. Prolonged incubations (72 hours) allowed the organisms to overcome the initial lag in many cases and obscured the effect shown in 24 hours.

5. Alterations of the Landy and Dicken medium with regard to inorganic content permitted higher acid production by *L. casei* and *L. delbrueckii* than the original formula. The modifications were not favorable to *L. arabinosus*.

REFERENCES

1. SNELL, E. E., *J. Bact.* **50**, 373 (1945).
2. MÖLLER, E. F., *Z. physiol. Chem.* **260**, 246 (1939).
3. WOOLLEY, D. W., *J. Biol. Chem.* **140**, 311 (1941).
4. BENTLEY, O. G., SNELL, E. E., AND PHILLIPS, P. H., *ibid.* **170**, 343 (1947).
5. MACLEOD, R. A., AND SNELL, E. E., *ibid.* **170**, 351 (1947).
6. LANDY, M., AND DICKEN, D. M., *J. Lab. Clin. Med.* **27**, 1086 (1942).
7. FRIEDEMANN, T. E., AND GRAESER, J. B., *J. Biol. Chem.* **100**, 291 (1933).
8. SOMOGYI, M., *ibid.* **117**, 771 (1937).
9. SANDELL, E. B., *Colorimetric Determination of Traces of Metals*. Interscience Publishers, Inc., New York, 1944.
10. PARKS, R. Q., HOOD, S. L., HURWITZ, C., AND ELLIS, G. H., *Ind. Eng. Chem., Anal. Ed.* **15**, 527 (1943).
11. KITZES, G., ELVEHJEM, C. A., AND SCHUETTE, H. A., *J. Biol. Chem.* **155**, 653 (1944).
12. CALVERY, H. O., *Food Research* **7**, 313 (1942).

VITA

The writer was born in Neenah, Wisconsin on April 14, 1921. She attended elementary and high schools in Fond du Lac County in that state. She graduated from Fond du Lac High School as Salutatorian of the class of 1937. She was awarded a scholarship to Ripon College and attended that institution for one year. In 1938 she transferred to the University of Wisconsin to major in Bacteriology.

She was awarded the Ida M. Suyvers scholarship during the year 1939-1940 and a Wisconsin Alumni Research Apprenticeship for 1940-1941. She received a Bachelor of Arts degree with honors in June, 1941 and was awarded a scholarship for graduate work by the Regents of the University.

While pursuing work toward the Master's degree, she held an appointment as Teaching Assistant in Bacteriology. The degree was granted in January, 1943. The remaining six months of the academic year she spent as a research assistant on a Wisconsin Alumni Research Foundation project.

In June, 1943, she enrolled in the graduate school of the University of Illinois. She has held the position of Teaching Assistant in Bacteriology from that time to the present.

She is a member of Sigma Xi and Sigma Delta Epsilon. Her publications are the following:

A Chemical and Immunological Study of the Capsular Polysaccharide of *Clostridium perfringens*. Jour. Bact. 48; 31-44, 1944 (with Elizabeth McCoy)

Bovine Intracutaneous and Serological Reactions to Fractions of *Trichomonas foetus* (Protozoon). Jour. Bact. 47: 505-508, 1944

